acetic acid (3:5). The chromatogram showed a large symmetrical peak at R_f 0.32, corresponding to the position established for the dimers of oxytocin.[3] Isolation of the material from the eluates represented by this peak gave 18.3 mg of the synthetic material. The product was dissolved in 0.2 *N* acetic acid (1.0 ml) and subjected to gel filtration on a 1.38 × 82 cm column in 0.2 *N* acetic acid. A flow rate of 5 ml/hr was maintained and the eluates were collected in fractions of 1.04 ml. Only one sharp peak was obtained with a maximum at effluent volume 85.6 ml, corresponding to the position for the dimers of oxytocin under the same conditions. Isolation by lyophilization gave 14.1 mg of the synthetic parallel dimer.

A sample of the parallel dimer was hydrolyzed in 6 *N* HCl at 110° for 24 hr and analyzed[13] on a Beckman-Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were found, with the value of leucine taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 0.9; leucine, 1.0; isoleucine, 1.0; tyrosine, 0.9; and ammonia, 3.1. Paper chromatography of the material on Whatman No. 1 in 1-butanol–acetic acid–water (5:1:5) gave only one spot, R_f 0.28, when color was developed with Pauly reagent, as compared with previously reported values of 0.28 and 0.23 for α dimer and β dimer, respectively.[3] On paper electrophoresis in 0.1 *M* sodium acetate buffer at pH 5.5 (18 hr at 4° and 300 V) the material migrated as one spot toward the cathode (color development with Pauly reagent).

Comparisons of Parallel Dimer with α Dimer. Subjection of the parallel dimer (0.83 mg) to gel filtration on a 1.24 × 159-cm column in 0.2 *N* acetic acid at a flow rate of 5.7 ml/hr gave a chromatogram showing only one sharp symmetrical peak with a maximum at effluent volume 127 ml, identical with that reported for the α dimer under these conditions.[3] The β dimer has a maximum at effluent volume 137 ml.[3] Gel filtration of a mixture of the parallel dimer (0.51 mg) and the α dimer (0.56 mg) gave a chromatogram showing only one sharp symmetrical peak with a maximum at effluent volume 127 ml. The parallel dimer exhibited the specific rotation $[\alpha]^{19}D$ −105° (*c* 0.5, 1 *N* acetic acid) as compared to $[\alpha]^{20}D$ −106° (*c* 0.5, 1 *N* acetic acid) reported for the α dimer. The β dimer exhibits the specific rotation $[\alpha]^{20}D$ −78° (*c* 0.5, 1 *N* acetic acid).[3] It is interesting that both the parallel dimer and the α dimer are readily soluble in 6 *N* HCl at room temperature; the β dimer is not soluble under these conditions.[3]

Conversion of Parallel Dimer to Oxytocin. A sample (7.24 mg, 3.40 μmol) of the parallel dimer was dissolved in 25 ml of liquid ammonia (distilled from sodium) and treated at the boiling point with sodium until a blue color persisted throughout the solution for 1 min. The solution was evaporated *in vacuo* to low volume and lyophilized. The residue was dissolved in deaerated water (30 ml) containing 0.1 *N* trifluoroacetic acid (0.30 ml). The sulfhydryl content of the solution was 11.1 μmol. The pH of the solution was adjusted to 7.5 with 0.1 *N* trifluoroacetic acid. The solution was aerated for 2.3 hr; no thiols were detected after this period. The solution was acidified with glacial acetic acid and lyophilized.

The product was dissolved in 2 ml of the organic phase of 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and subjected to partition chromatography in this solvent system under the following conditions: column size, 0.95 × 55.05 cm; hold-up volume, 10.3 ml; fraction volume, 1.07 ml; flow rate, 2.8 ml/hr; regenerating solvent, pyridine–0.1% aqueous acetic acid (3:5). The chromatogram showed a major peak with R_f 0.28 corresponding to the position of oxytocin under these conditions. Isolation of the oxytocin gave 3.2 mg possessing an oxytocic potency[14] of about 540 units/mg.

Acknowledgments. We wish to express our gratitude to Professor Vincent du Vigneaud for his support, advice, and encouragement. We also wish to thank Mr. Joseph Albert for the elemental analyses and Miss Margitta Wahrenburg and Mrs. Jessie Lawrence for the bioassays performed under the direction of Dr. W. Y. Chan.

(14) The assays for oxytocic activity were performed on isolated rat uteri from rats in natural estrus according to the method of P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948), as modified by R. A. Munsick, *Endocrinology*, **66**, 451 (1960), with the use of magnesium-free van Dyke–Hastings solution. Oxytocic activity was measured against the USP posterior pituitary reference standard.

Communications to the Editor

Chemistry of Singlet Oxygen. X. Carotenoid Quenching Parallels Biological Protection[1]

Sir:

β-Carotene efficiently quenches singlet oxygen, generated either by dye sensitization or by the NaOCl–H_2O_2 reaction.[2] This quenching bears on the mechanism of the protective action of carotenoids against photodynamic damage in living organisms; this protective action may be the "universal function of carotenoid pigments."[3]

We now report that *the rate of quenching is a sensitive function of the length of the conjugated polyene chain and parallels the protective action of natural compounds.* The techniques used were similar to those previously reported.[2,4] The compounds used as quenchers were all-*trans*-retinol (**1**, 5 conjugated C=C) and two synthetic carotene analogs, a C_{30} hydrocarbon (**2**, 7 conjugated C=C) and a C_{35} hydrocarbon (**3**, 9 conjugated C=C).[5]

In the previous paper, it was shown that when $[AO_2]^{-1}$ is plotted against $[A]^{-1}$ at constant [Q], straight lines result; the intercept on the $[AO_2]^{-1}$ axis is a measure of the amount of singlet oxygen formed, and the increase in ratio of slope to intercept compared to plots with no Q is a measure of quenching of singlet oxygen (a kinetic scheme for this system is given in the accompanying communication).[6] Thus quenching of

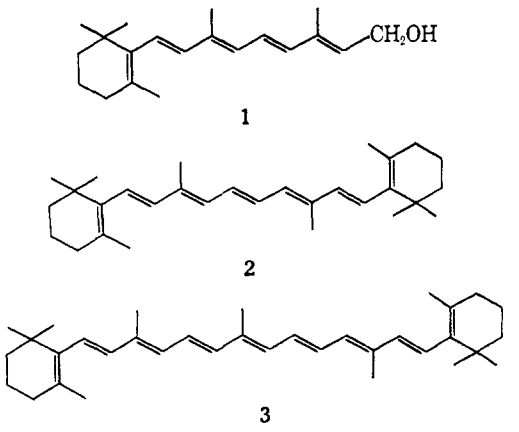
(1) Paper IX: S. Mazur and C. S. Foote, *J. Amer. Chem. Soc.*, **92**, 3225 (1970). Contribution No. 2557; supported by a grant from the USPHS-NAPCA (No. AP-00681).

(2) C. S. Foote and R. W. Denny, *J. Amer. Chem. Soc.*, **90**, 6233 (1968); C. S. Foote, R. W. Denny, L. Weaver, Y. Chang, and J. Peters, *Ann. N. Y. Acad. Sci.*, in press.

(3) N. I. Krinsky, *Photophysiology*, **3**, 123 (1968).

(4) Solutions in benzene–methanol (80:20) containing known amounts of the photooxygenation acceptor 2-methyl-2-pentene (A) and the quencher (Q) were irradiated for a constant time, sufficient to produce a readily measurable amount of product (AO_2) without oxidizing a significant fraction of A (<7%). The sensitizer was methylene blue (MB); solutions under pure O_2 were irradiated with a tungsten–halogen lamp through a $K_2Cr_2O_7$ filter (3 g/100 ml of H_2O, 2.5-cm path length) with shortwave cutoff (1% transmission) of 510 nm, which ensured that light was absorbed only by the sensitizer, not by the quencher. Although this filter was not used in previous experiments,[2] controls and subsequent experiments with filtered light established that light absorbed by β-carotene had no effect on the observed quenching or on the production of AO_2. Photooxygenated solutions were reduced with $NaBH_4$, internal standard was added, and the product alcohols were determined gas chromatographically.

(5) The C_{30} and C_{35} hydrocarbons were kindly supplied by Dr. H. Pommer, BASF, Ludwigshafen, Germany. For the purposes of this discussion, the endocyclic double bonds in all carotenoids are counted as conjugated.



triplet sensitizer but not singlet oxygen gives an increase in intercept, but no change in slope/intercept; quenching of singlet oxygen but not of sensitizer gives an increase only in slope, and the intercept remains constant. β-Carotene showed the latter behavior.[2]

The behavior of the present three compounds shows a dramatic change with chain length: whereas β-carotene is an effective inhibitor of photooxidation at 10^{-4} *M*, **3** is an order of magnitude less effective; however, both of these compounds quench singlet oxygen at concentrations well below those at which triplet sensitizer could be quenched, and no increase in intercept is observed at the highest concentrations used (10^{-4} *M* for β-carotene and 8×10^{-4} *M* for **3**). Compound **2** is less efficient as a quencher, and both singlet oxygen and triplet sensitizer are quenched (at 9×10^{-3} *M*, there is a fivefold increase in intercept and a fourfold increase in slope/intercept ratio). With retinol at 6.7×10^{-3} *M*, the intercept increased by a factor of 3, but there was no detectable increase in slope/intercept, so that only triplet quenching is detectable.[7] Under the conditions of the experiment, none of the quenchers was destroyed appreciably, although a small amount of retinol (~10%) was lost by spectrophotometric assay.[9]

Table I. Quenching Parameters for Carotenoid Inhibition of 2-Methyl-2-pentene Photooxygenation, Sensitized by Methylene Blue

Quencher	k_Q/k_A[a] (singlet oxygen)	k_Q^T, M^{-1} sec^{-1} [a] (^{3}sensitizer)
1	≤9[c]	4×10^9 [d]
2	57	7×10^9 [d]
3	1900	[e]
β-Carotene[b]	10^4	[e]

[a] See ref 6 for kinetic scheme. [b] Reference 2. [c] Upper limit, twice the probable error in the determination. [d] Assumes $[O_2] = 10^{-2}$ *M* and O_2 quenching rate 1.2×10^9 M^{-1} sec^{-1}; see ref 10. [e] Not measurable at concentrations used.

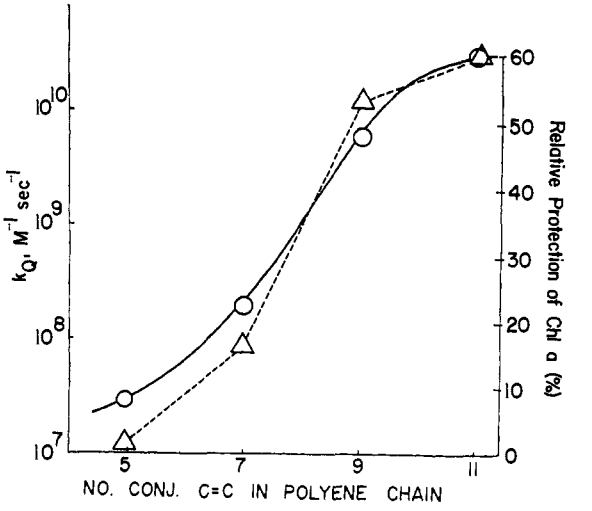


Figure 1. $^1O_2$ quenching rates (k_Q, ○—○) and protective action against photobleaching of chlorophyll a[11] (△---△) as a function of length of conjugated system.

The kinetic parameters[6] determined for the three new quenchers are listed in Table I, with the value for β-carotene[2] for comparison. The ratio k_Q/k_A is calculated from the increase in slope/intercept, and is the ratio of the rate at which singlet oxygen is quenched (k_Q) to its reaction rate with A (k_A, see ref 2); the value k_Q^T is the rate of quenching of triplet methylene blue, estimated from the increase in intercept. The parameters are estimated to have a probable error of a factor of 1.5 because the intercept values are difficult to determine accurately.

If the singlet oxygen quenching by β-carotene is assumed to be diffusion controlled, and a rate of 3×10^{10} M^{-1} sec^{-1} is assigned,[10] the quenching rates for all quenchers can be calculated. These rates are plotted against the number of conjugated double bonds in Figure 1. It is immediately obvious that the rates fall off sharply with decreasing chain length, the sharpest drop occurring between 7 and 9 conjugated double bonds.[10a]

This behavior is remarkably similar to the protective action of natural carotenoids against chlorophyll photobleaching[11] (also shown in Figure 1) which also falls off most sharply between 7 and 9 conjugated double bonds. This protection against chlorophyll photobleaching is one of the mechanisms by which carotenoids protect against photodynamic damage in living systems.[3,11] The similarity of the effects suggests that (1) carotenoids protect living organisms from photodynamic damage at least partly by quenching singlet oxygen and (2) singlet oxygen is at least one cause of photodynamic damage. Although enzymes, nucleic

(6) C. S. Foote, Y. C. Chang, and R. W. Denny, *J. Amer. Chem. Soc.*, **92**, 5218 (1970).

(7) Both retinol and β-carotene are reported to quench triplet sensitizers at a high rate;[8] it is presumed that all four compounds used here quench triplet methylene blue efficiently; however, this quenching, even if diffusion controlled, cannot compete with oxygen quenching of triplet methylene blue below about 10^{-3} *M*.

(8) R. Livingston and A. C. Pugh, *Discuss. Faraday Soc.*, **27**, 144 (1959); E. Fujimori and R. Livingston, *Nature (London)*, **180**, 1036 (1957); A. Sykes and T. G. Truscott, *Chem. Commun.*, 929 (1969).

(9) Quenching values were also determined for lycopene (11 C=C) and β-*apo*-8′-carotenol (9 C=C) and found to be similar to those for β-carotene and **3**, respectively, but the rates could not be determined accurately because substantial destruction of these carotenoids occurred under the conditions.

(10) The rate of O_2 quenching of naphthalene fluorescence in benzene: W. R. Ware, *J. Phys. Chem.*, **66**, 455 (1962). The rate used in ref 2 was an O_2 *triplet* quenching rate, and is too low because of a spin-statistical factor of 9. The absolute rate of $^1O_2$ decay in solution is calculated, based on this rate, to be 1.0×10^{-5} sec^{-1} and is a lower limit. The rate of triplet quenching by O_2 used in the calculation, 1.2×10^9 M^{-1} sec^{-1}, was taken from K. Gollnick, *Advan. Photochem.*, **6**, 1 (1968).

(10a) NOTE ADDED IN PROOF. Quenching rates for a C_{50} and a C_{60} carotenoid (15 and 19 conjugated C=C) are essentially the same as that of β-carotene, confirming the suggestion that the latter rate is diffusion controlled.

(11) (a) H. Claes, *Biochem. Biophys. Res. Commun.*, **3**, 585 (1960); (b) H. Claes and T. O. M. Nakayama, *Z. Naturforsch. B*, **14**, 746 (1959).

acids, and cell membranes are also damaged in photodynamic action, carotenoids also protect against these effects,[3] presumably by the same mechanism.

The behavior of these carotenoids in triplet MB quenching also serves to explain the fact that shorter carotenoid chains are effective in protecting against *anaerobic* chlorophyll photoreduction, since triplet chlorophyll a is probably intermediate in this reaction, and quenching of this triplet is effective even with carotenoids of only 5 conjugated double bonds.[8]

The implications of these observations for the mechanism of singlet oxygen quenching by carotenoids are discussed in the accompanying communication.[6]

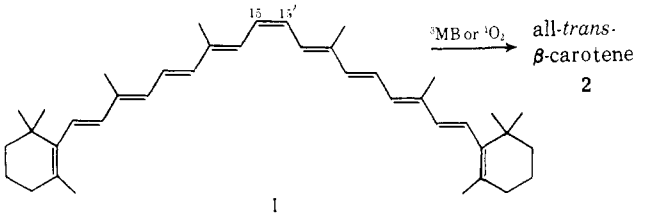
* To whom correspondence should be addressed.

Christopher S. Foote,* Yew C. Chang, Robert W. Denny
Department of Chemistry, University of California
Los Angeles, California 90024
Received March 19, 1970

Chemistry of Singlet Oxygen. XI. Cis–Trans Isomerization of Carotenoids by Singlet Oxygen and a Probable Quenching Mechanism[1]

Sir:

Numerous reports of cis–trans isomerization of carotenoids by direct or sensitized irradiation have appeared.[2] The isomerization of carotenoids sensitized by chlorophyll a appears to occur only in the direction cis → trans.[2,3] We now report that isomerization of 15,15′-*cis*-β-carotene (**1**) is sensitized by singlet oxygen ($^1O_2$), as well as by triplet methylene blue (^{3}MB) with high efficiency. The product is, at least largely, all-*trans*-β-carotene (**2**). Under the same conditions, **2** does not appear to undergo appreciable isomerization.



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The apparatus and conditions used are similar to those used for the quenching experiments,[1,4] except that 2-methyl-2-pentene was omitted from most experiments (having been found not to affect the results), and that changes in carotene composition were monitored by absorption spectroscopy.[5] All-*trans*-β-carotene has strong (ε 114,000) absorption at 464 nm and only weak (ε 7500) absorption at 345 nm; 15,15′-*cis*-β-carotene has weaker absorption at 456 nm (ε 85,000) but much stronger absorption at 345 nm (ε 49,400); this latter absorption is referred to as the "cis peak" and is much stronger for the symmetrical 15,15′-cis isomer than for other *cis*-carotenes.[2a]

Solutions of **1** and methylene blue (MB) were irradiated under O_2 through a filter[1] which ensured that light was absorbed only by MB. The results are summarized in Table I, and show a rapid decrease in the "cis peak" and a simultaneous increase in absorbance at 464 nm. The results are consistent with a clean isomerization **1** → **2**; the absorbance at 464 nm calculated from the decrease at 345 nm on the assumption that no other reaction occurs is in excellent agreement with the observed values. Reaction under N_2 was equally rapid, but no reaction whatever occurred under O_2 without MB.

Table I. Absorbance of a Solution of 15,15′-*cis*-β-Carotene[a] and Methylene Blue[b,c]

Irradn time, sec	Absorbance 345 nm	Absorbance 464 nm	Isomerization, %[d]	A_{464}, calcd[e]
0	0.68	1.39	14	1.40
3	0.49	1.54	43	1.53
6	0.39	1.66	58	1.60
9	0.28	1.70	76	1.68
12	0.19	1.77	89	1.74

[a] **1**, 1.57×10^{-4} *M*. [b] 4×10^{-5} *M*. [c] In C_6H_6:CH_3OH (80:20); irradiated through $K_2Cr_2O_7$ filter; see ref 1. [d] Calculated from decrease in absorbance at 345 nm. [e] Calculated on assumption that reaction **1** → **2** is quantitative.

The carotene was isolated (in 80% yield) from an isomerization run under the conditions of the quenching experiments[1] (containing 2-methyl-2-pentene) by Ca(OH)$_2$ chromatography, and was found to be pure all-*trans*-β-carotene by melting point and mixture melting point, R_f, uv–visible spectrum, and ir spectrum (absence of cis band[6] at 772 cm^{-1}). Other cis isomers were shown to be absent by tlc (isomerization of **2** with I_2 produces a mixture which contains several other cis isomers which are readily separable by chromatography[7]). Similar experiments using all-*trans*-β-carotene gave no isomerization. From the amount of 2-methyl-2-pentene peroxide produced in photooxygenation experiments[1,4] and the rate of isomerization, it can be calculated that each singlet oxygen molecule produced causes isomerization of slightly less than one molecule of **1** to **2**.

A similar series of experiments was carried out using $^1O_2$ generated from NaOCl–H_2O_2[8] in C_6H_6:CH_3OH:diglyme (1:1:1);[4] all experiments were carried out in darkness to avoid photoisomerization of carotenes. As in the photochemical experiments, **1** underwent smooth isomerization to the all-trans isomer (identified as above), but **2** was not isomerized detectably under identical conditions. No isomerization of **1** occurred on addition of spent reagents nor of H_2O_2 alone. The initial efficiency of isomerization

(1) Paper X: C. S. Foote, Y. C. Chang, and R. W. Denny, *J. Amer. Chem. Soc.*, **92**, 5216 (1970). Contribution No. 2570; supported by a grant from the USPHS-NAPCA (No. AP-00681).

(2) (a) L. Zechmeister, "*Cis-trans*-Isomeric Carotenoids, Vitamins A and Arylpolyenes," Academic Press, New York, N. Y., 1962, pp 46–57; (b) H. Claes and T. O. M. Nakayama, *Nature (London)*, **183**, 1053 (1959); (c) H. Claes, *Biochem. Biophys. Res. Commun.*, **3**, 585 (1960); (d) H. H. Inhoffen, F. Bohlmann, K. Bartram, G. Rummert, and H. Pommer, *Justus Liebigs Ann. Chem.*, **570**, 54 (1950).

(3) L. Zechmeister, A. LeRosen, W. A. Schroeder, A. Polgár, and L. Pauling, *J. Amer. Chem. Soc.*, **65**, 1940 (1943).

(4) C. S. Foote and R. W. Denny, *ibid.*, **90**, 6233 (1968).

(5) β-Carotene (Eastman Organic Chemicals) was recrystallized from benzene–methanol (mp 180–181°). 15,15′-*cis*-β-Carotene was kindly supplied by Dr. U. Schwieter, Hoffmann-LaRoche, Basel, and had mp 145°, with resolidification and melting at 167–170°. (The material as used subsequently had undergone some (~10%) isomerization to all-*trans*-β-carotene.)

(6) O. Isler, *Helv. Chim. Acta*, **39**, 249 (1956).

(7) A. Polgár and L. Zechmeister, *J. Amer. Chem. Soc.*, **64**, 1865 (1942).

(8) C. S. Foote, S. Wexler, W. Ando, and R. Higgins, *ibid.*, **90**, 975 (1968).